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Identification of a Novel Tripartite Complex Involved in Replication of Vesicular Stomatitis Virus Genome RNA

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Our laboratory's recent observations that transcriptionally inactive phosphoprotein (P) mutants can efficiently function in replicating vesicular stomatitis virus (VSV) defective interfering particle in a three-plasmid-based (L, P, and N) reverse genetics system in vivo (A. K. Pattnaik, L. Hwang, T. Li, N. Englund, M. Mathur, T. Das, and A. K. Banerjee, J. Virol. 71:8167-8175, 1997) led us to propose that a tripartite complex consisting of L-(N-P) protein may represent the putative replicase for synthesis of the full-length genome RNA. In this communication we demonstrate that such a complex is indeed detectable in VSV-infected BHK cells. Furthermore, coexpression of L, N, and P proteins in Sf21 insect cells by recombinant baculovirus containing the respective genes also resulted in the formation of a tripartite complex, as shown by immunoprecipitation with specific antibodies. A basic amino acid mutant of P protein, P260A, previously shown to be inactive in transcription but active in replication (T. Das, A. K. Pattnaik, A. M. Takacs, T. Li, L. N. Hwang, and A. K. Banerjee, Virology 238:103-114, 1997) was also capable of forming the mutant [L-(N-Pmut)] complex in both insect cells and BHK cells. Sf21 extract containing either the wild-type P protein or the mutant P protein along with the L and N proteins was capable of synthesizing 42S genome-sense RNA in an in vitro replication reconstitution reaction. Addition of N-Pmut or wild-type N-P complex further stimulated the synthesis of the genome-length RNA. These results indicate that the transcriptase and replicase complexes of VSV are possibly two distinct entities involved in carrying out capped mRNAs and uncapped genome and antigenome RNAs, respectively.

Vesicular stomatitis virus (VSV), the prototype rhabdovirus, contains a single-stranded negative-sense genome RNA consisting of 11,161 nucleotides (nt) (26, 27) which is encapsidated by the nucleocapsid protein (N) and associated with the RNA-dependent RNA polymerase consisting of a large protein (L) and the phosphoprotein (P) along with specific host factors (1). In the transcription process, phosphorylation of P protein by cellular casein kinase II at specific sites is required for its activity (3, 4, 5). The virion-associated RNA polymerase synthesizes sequentially from the 3′ end of the genome RNA, a leader RNA (47 nt long) and five mRNAs in order, both in vitro and in vivo, encoding the N, P, M (matrix protein), G (glycoprotein), and L proteins. During the replication reaction, however, a critical attenuation of transcription occurs at the gene junctions, resulting in the synthesis of a full-length antigenome-sense RNA which is concomitantly enwrapped with the N protein. The positive-sense N-RNA complex then serves as a template for the RNA polymerase to produce more genome-sense progeny N-RNA, leading to mature virus synthesis. Since the encapsidation by N protein is a prerequisite during the replication process (1, 7, 12, 15, 22, 23), it has been proposed that the N protein somehow modifies the RNA polymerase, thus facilitating the attenuation process (1, 7). It was subsequently shown that the N protein in the infected cells remains complexed with the P protein (6, 24), and this soluble complex, not N by itself, is directly involved in the replication process. It is also generally believed that the RNA polymerase, which is composed of L and P proteins, carries out both the RNA synthetic events, i.e., transcription and replication, with N-P complex initiating the latter process. The exact mechanism of the replication reaction, however, remains unclear.

Some recent observations from our laboratory have brought to light a possible scenario where the transcriptase and replicase may, in fact, be two distinct entities. Using a reverse genetics system and utilizing a transcribing minigenome and defective interfering particle, we have shown that both phosphorylation-negative mutants (21) as well as mutation at the C-terminal basic domain of the P protein (11) are transcriptionally inactive but are fully active in replication of a defective interfering particle. These mutants, however, are capable of forming the soluble N-P complex, the required intermediate for the replication reaction (28). Since these mutant P proteins (Pmut) cannot provide the transcriptive function to the L protein but support replication, it was speculated that the L protein must interact directly with the N-P complex to form a functionally active tripartite complex L-(N-Pmut) which may serve as the putative replicase to carry out the replication reaction. These studies raised the possibility that replicase and transcriptase are two distinct entities with distinctly different subunit compositions.

To gain insight into the structure and function of the putative tripartite complex, we first studied the interaction of L, P, and N proteins both from VSV-infected mammalian cells as well as from insect cells expressing both wild-type and mutant recombinant proteins. BHK 21 cells were first infected with VSV Indiana serotype at a multiplicity of infection of 10.0. Infected cells were labeled with [^{35}S]methionine for 1 h before

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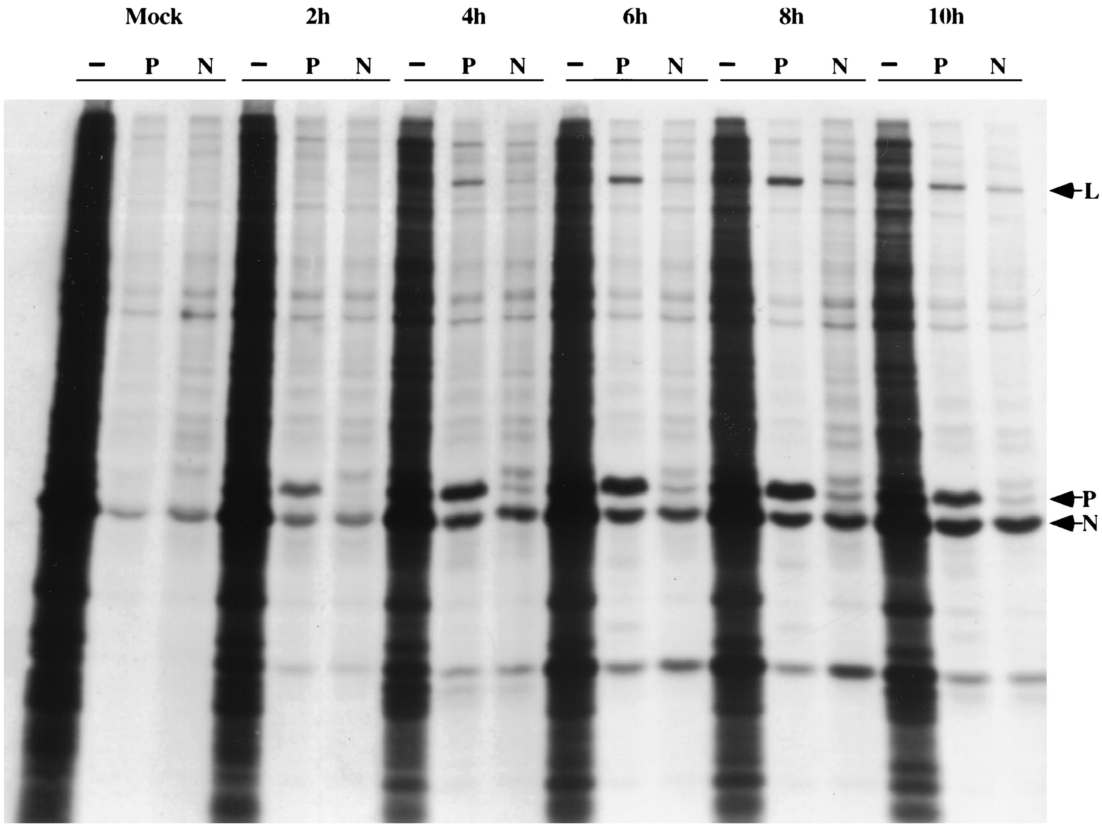

FIG. 1. Association of L and N protein in VSV-infected BHK 21 cells. BHK 21 cells, infected with VSV at a multiplicity of infection of 10.0, were incubated for different time periods postinfection followed by 1 hour of labeling with [^{35}S]methionine. Cells were then lysed in a hypotonic buffer containing 20 mM Tris (pH 7.5), 20 mM NaCl. After lysis, the extract was adjusted to a final concentration of 150 mM NaCl, 1 mM dithiothreitol, 5% glycerol, followed by centrifugation at 100,000 × *g*. The soluble supernatant (S-100) was immunoprecipitated either with P antibody (P) or with N antibody (N). Numbers above the lanes indicate the time (in hours) postinfection that samples were collected. Mock, uninfected cell extract; −, samples before immunoprecipitation. The arrows on the right side indicate the position of migration of the different viral proteins.

harvesting at different time periods postinfection. To score for the interacting viral proteins, soluble supernatant was immunoprecipitated with either P or N antibody (Fig. 1). As expected, the L and the N proteins were immunoprecipitated by P antibody; these two proteins are known to interact independently with the P protein. Interestingly, using N antibody, L protein was precipitated along with the P protein, suggesting that the L protein must have interacted with the N protein; the latter is possibly complexed with the P protein. The formation of such a tripartite complex was discernible at 4 h and increased at later time periods postinfection. It is important to note that the amount of L and P proteins pulled down from the extract by the N monoclonal antibody is quite different compared to the complex precipitated by the P antibody. Although the amount of N protein was identical in both cases, the P protein content was disproportionately low in the complex pulled down by the N antibody (Fig. 1). In contrast, P antibody was able to pull down a significant amount of N protein, commensurate with its forming a complex with the P protein. Although the reason for this difference in N protein content in these complexes is unclear, it should be noted that the N antibody is a monoclonal antibody which may interact with a specific epitope displayed by the tripartite L-N-P complex. The fact that a significantly higher amount of L protein was precipitated by the P antibody than by the N antibody indicates that two different forms of complexes, e.g., L-P and L-N-P, may have been formed, both of which are precipitable by the P antibody. Finally, these observations indicate that in VSV-infected cells, the L and N proteins appear to interact and form a complex in the presence of the P protein. Note that a low-molecular-weight band appeared in every lane in the immunoprecipitate of the virus-infected extracts. This band appears to be either a degradation product or an internally initiated fragment of the N protein.

Because it was not possible to determine from the above studies whether the L-N interaction can take place in the absence of P, we used recombinant baculoviruses to study the interaction of expressed recombinant proteins in insect cells (19). Since the baculovirus expression system is known to be an efficient one, we initially used Sf21 cells for infection with recombinant baculovirus containing either the L or P or N gene. Seventy-two hours postinfection, soluble cell extracts were prepared and the syntheses of the recombinant proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by Coomassie blue staining. As shown in Fig. 2A, each protein was efficiently expressed

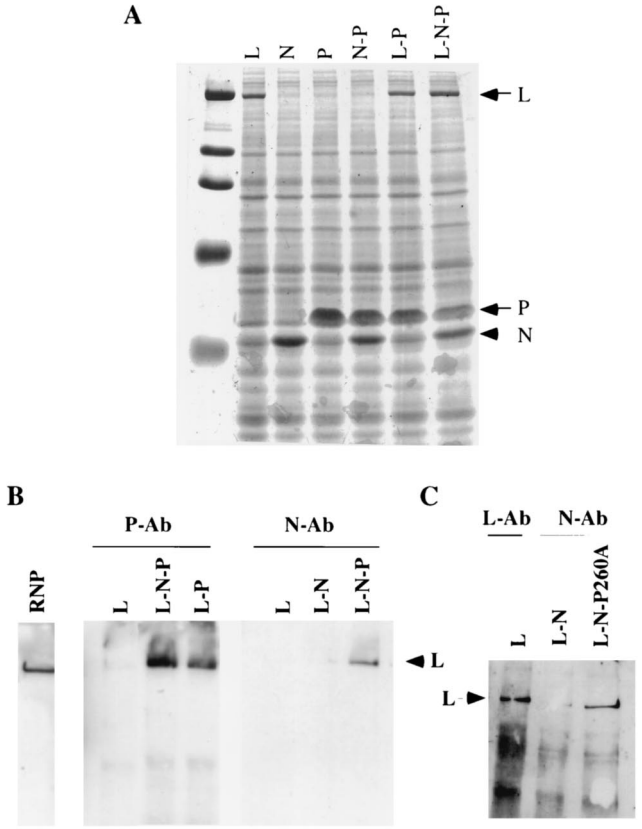

FIG. 2. Expression and association of the recombinant proteins in Sf21 cells. Sf21 cells, grown in a suspension culture, were infected singly, doubly, or triply with recombinant baculovirus containing wild-type L, P, and N genes at a multiplicity of infection ratio of 1:5:5 (24). Seventy-two hours postinfection, the cells were harvested and the soluble extract (S-100) was made as described in the legend for Fig. 1. (A) A 20-μg aliquot of total protein for each sample was analyzed in an SDS-polyacrylamide gel followed by Coomassie blue staining. Recombinant viruses used to infect the cells are marked on the top of each lane. The position of migration of the L, P, or N protein is indicated by the arrow. (B and C) Immunoprecipitation of Sf21 soluble (S-100) extracts. Cells expressing different viral proteins either alone or in combinations, marked on top of each lane, were immunoprecipitated either with P antibody (P-Ab), N antibody (N-Ab), or L antibody (L-Ab) followed by Western blotting with anti-L antibody. (B) All three expressed proteins are wild type. The extreme left lane (RNP) indicates the position of migration of the L protein using viral RNP as an authentic source, detected by Western blotting. (C) Results of the same experiment as shown in panel B, with the exception that instead of the wild-type P protein cells were infected with a mutant construct, P260A.

when Sf21 cells were infected singly or in combination with recombinant baculoviruses. Each extract was then subjected to immunoprecipitation using either P or N antibody followed by immunoblotting with L antibody (Fig. 2B). While the P antibody can coprecipitate the L protein either from L-P- or L-P-N-expressing extracts, the N antibody again brought down the L protein only when all three proteins were expressed. Interestingly, the L protein failed to interact with the N protein in the absence of the P protein. Thus, it seems that the L protein does indeed form a tripartite complex composed of N and P only when all three proteins are coexpressed in insect cells.

Similar to the wild-type P protein, P260A was also found to be active in forming the tripartite complex with the L and N proteins. As shown in Fig. 2C, the L protein was pulled down with N antibody only when all three proteins were coexpressed, indicating the formation of an L-(N-Pmut) complex. In contrast, no L was seen when L and N were coexpressed followed by immunoprecipitation with N antibody. Thus, it seems that the mutant P protein is also capable of forming a tripartite complex with L and N, similar to the wild-type P protein.

To further confirm that the L-N interaction is dependent on the presence of the P protein, we used rabbit reticulocyte lysate to translate in vitro the N protein alone or in the presence of wild-type (Pwt) or mutant (P260A) P protein. After translation, the postribosomal supernatant was incubated with partially purified recombinant L protein expressed in insect cells (10), followed by immunoprecipitation with anti-L antibody. As shown in Fig. 3B, L antibody efficiently pulled down both N and the wild-type or mutant P proteins when expressed together. In contrast, no specific immunoprecipitation of N protein by the L antibody could be detected when N protein was expressed alone and incubated with L protein. Note that the amount of N protein that cross-reacted with L antibody in the absence of L protein is virtually identical to that observed in the presence of L protein. These results further confirm that N protein cannot interact with L protein in the absence of P protein.

Next, we studied the RNA-synthetic ability of the Sf21-expressed putative tripartite complex in a reconstituted transcription-replication reaction in vitro. Following incubation with the N-RNA template and ribonucleoside triphosphates, the product RNAs were analyzed by electrophoresis in a 1% agarose formaldehyde gel. As shown in Fig. 4, the coexpressed L-P extract efficiently transcribed the N-RNA template to synthesize mRNAs. The majority of the labeled products migrating below the 6.0-kb marker (Fig. 4A) are the mRNAs, as judged by the binding to an oligo(dT) cellulose column (data not shown). In contrast, when extracts containing all three proteins were added to the N-RNA template, synthesis of a genome-length RNA was clearly discernible. There was a significant increase in genome-length RNA synthesis when extract containing N-P complex was added under these conditions (Fig. 4A). Note that when extract expressing both the N and P proteins was further added to the L-P-expressing extract, the synthesis of the genome-length RNA did not increase significantly. These results indicate that the coexpression of all three proteins is required for efficient replication in vitro. Stimulation of replication by the addition of N-P-expressing extract is probably due to the requirement of N protein on a continuous basis for encapsidation of the nascent RNA synthesis. It appears then that in the triply expressed cell extract, the major complex is represented by the tripartite complex with little, if any, N-P complex. Note that the length of the heterogeneous RNAs (lane 4) is increased, which probably represents incomplete read-through RNA synthesized during the replication reaction.

To confirm the polarity as well as the identity of the ^{32}P-labeled RNA products synthesized in vitro migrating near the 11-kb region, Northern blot experiments were carried out (Fig. 4B and C). ^{32}P-labeled positive-sense or negative-sense riboprobe of the P gene was synthesized by T7 RNA polymerase or by SP6 RNA polymerase, respectively. In both cases, the P-

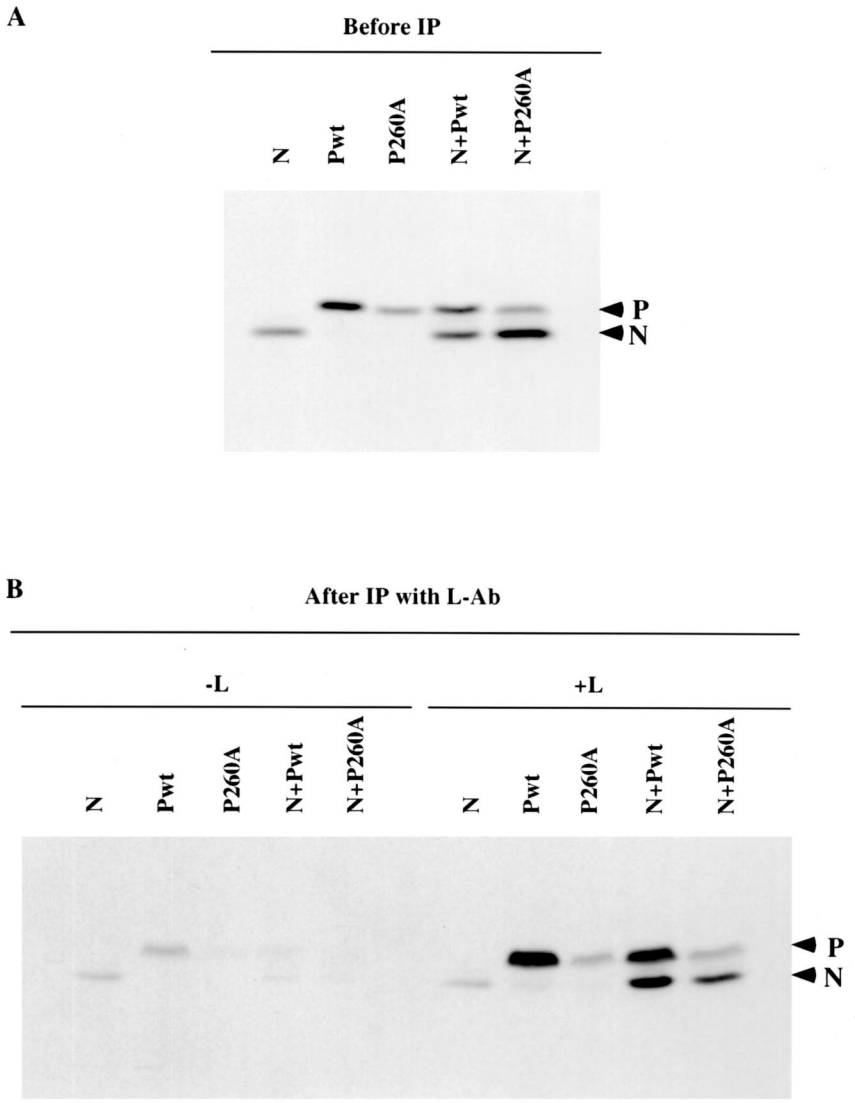

FIG. 3. Association of L, N, and P proteins in a cell-free translation system. In vitro-synthesized, T7-transcribed RNAs for N, Pwt, or the P260A mutant were added to a 40-μl rabbit reticulocyte lysate mixture either individually or in combination along with [^{35}S]methionine. The lysate was incubated for 90 min at 30°C followed by sixfold dilution with 150 mM NaCl containing Tris-EDTA (pH 8.0) buffer and centrifugation on a 30% glycerol cushion at 100,000 × *g* for 60 min. The supernatant (240 μl) was collected. (A) Ten microliters of each of the supernatants was directly analyzed in an SDS-polyacrylamide gel followed by autoradiography. (B) One hundred microliters of each of the supernatants was incubated at 30°C for 30 min either in the absence (-L) or presence (+L) of partially purified recombinant L expressed in insect cells (10 μg) and subsequently immunoprecipitated with anti-L antibody, followed by gel electrophoresis and autoradiography. The positions of migration of the N and P proteins are marked.

RNA probe hybridized with the newly synthesized RNAs migrating near the 11-kb region, indicating the synthesis of full-length positive-sense (Fig. 4C) and genome-sense (Fig. 4B) RNAs in vitro. The band appearing in the control lane (Fig. 4B, lane 1) represents the negative-sense genome RNA derived from the input template N-RNA.

Similarly, to determine the replicase activity of the tripartite complex containing the mutant P protein in vitro, partially purified fractions of L-P260A, L-(N-P260A, and N-P260A were used in a transcription-replication reaction in the presence of N-RNA template (Fig. 5). This purification step was necessary because the presence of some putative host factor(s) in crude cell extracts nonspecifically enhanced the transcription reaction, which would be otherwise defective in transcription (Fig. 5A and B, lanes 3 and 4). In fact, preliminary experiments (data not shown) strongly suggest that a cellular factor(s) may be responsible for activating the transcriptionally defective P260A mutant when it is expressed in insect cells. When the N-P260A complex (partially purified) was added to the L-(N-P260A)-containing fraction (partially purified), synthesis of genome RNA was significantly increased (Fig. 5B, lane 5), indicating that the N-P260A complex is capable of replicating the genome RNA similar to the wild-type complex (Fig. 4). Note that the transcription of mRNAs from L-P260A (partially purified) and N-RNA (Fig. 5A, lane 3) was also increased (Fig. 5A, lane 5) with the addition of the N-P260A

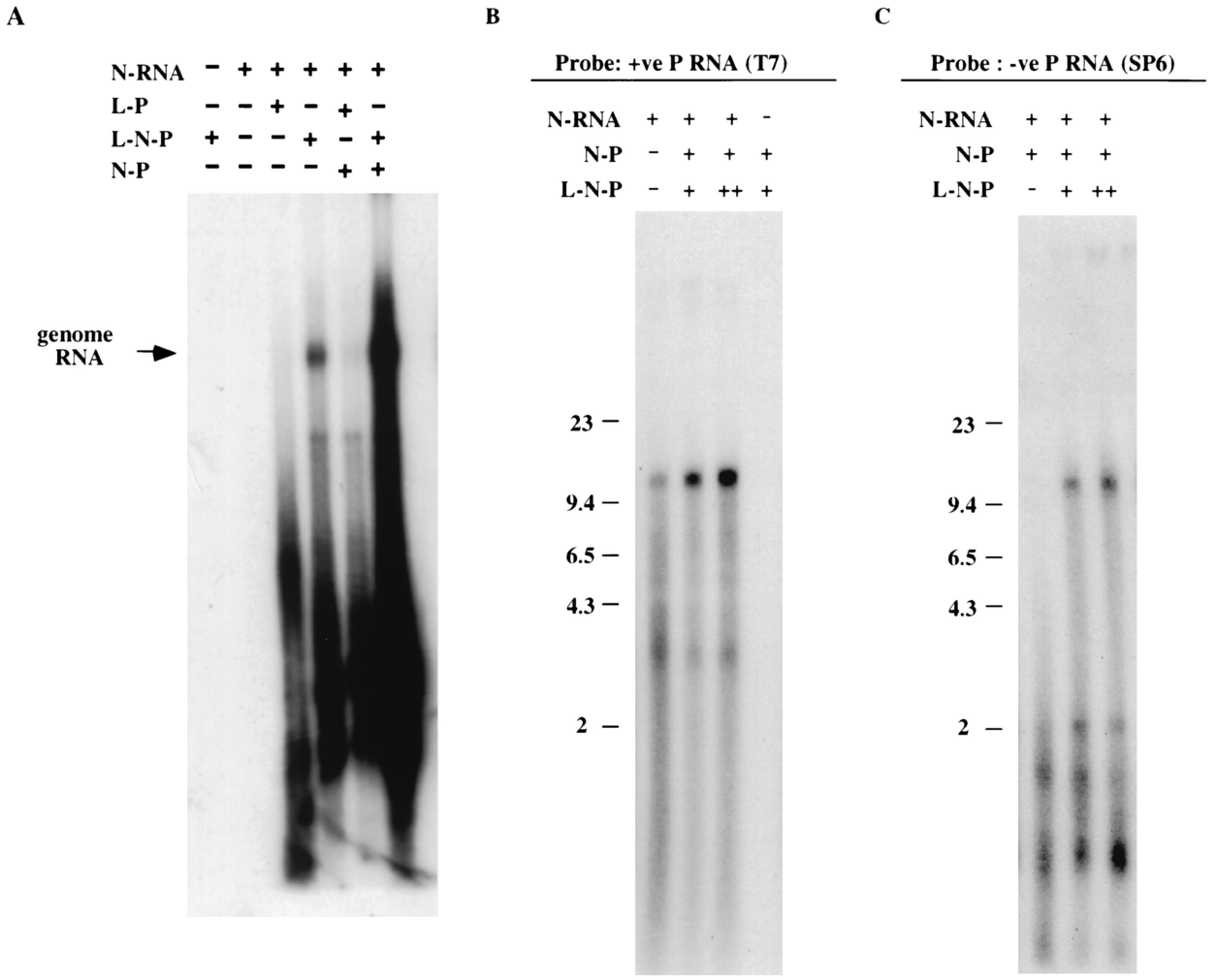

FIG. 4. Reconstitution of in vitro replication reactions using Sf21-expressed wild-type L, N, and P proteins. The in vitro replication reaction was carried out with (+) or without (−) N-RNA template in the presence (+) or absence (−) of Sf21 cell extracts expressing different recombinant proteins. (A) Analysis of the ^{32}P-labeled in vitro-synthesized RNA products in a 0.8% agarose–formaldehyde gel. N-RNA template isolated from purified VSV virions was incubated with the soluble supernatant (S-100) of Sf21-expressed proteins at 30°C for 3 h in a buffer containing 100 mM HEPES (pH 7.0), 150 mM NH_4Cl, 6 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM concentration each of ATP, CTP, and GTP, 100 μM UTP, 25 μCi of [α-^{32}P]UTP, and 80 U of RNasin/ml. The reaction mixtures were then deproteinized, followed by ethanol precipitation. The RNA samples were then denatured with a formamide-formaldehyde-containing denaturing dye and run in a formaldehyde-agarose gel. The position of 42S genome RNA is indicated by an arrow. (B and C) Northern blot analyses of unlabeled RNAs synthesized from an in vitro replication reaction either by hybridization with positive-sense (T7-transcribed) (B) or negative-sense (SP6) (C) P riboprobes. Numbers on the left side indicate the position of migration of known molecular mass markers (in kilobases).

complex, suggesting that the N-P260A fraction may still contain a cellular factor(s) responsible for nonspecific transcription activation. The synthesized genome RNA was confirmed to contain both 42S plus and minus strands, as shown in Fig. 4B and C and data not shown.

In this report, we present evidence for the first time that indeed a tripartite complex containing L, P, and N proteins is formed in VSV-infected cells (Fig. 1) as well as in insect cells when the three proteins are coexpressed (Fig. 2). The exact stoichiometry of the component proteins in the complex, however, remains undetermined at the present time. It is quite clear that in the absence of P protein, no interaction of L protein with N protein occurred, indicating that P protein is required to form the tripartite complex. This putative tripartite complex, irrespective of whether formed in the presence of wild-type or mutant P, initiates synthesis of a genome-length RNA in vitro in the presence of the N-RNA template (Fig. 4 and 5). Addition of the N-Pwt or N-P260A complex to the extract further stimulated the replication reaction, suggesting an important role for the N-P complex in the replication reaction, probably in providing N protein for encapsidation. The ability of the transcriptionally inactive P260A mutant to carry out the replication reaction in conjunction with the L and N proteins supports our contention that the replicase is structurally distinct from the transcriptase. Similar experiments using transcriptionally inactive P protein, e.g., P3A, are currently in progress. It is noteworthy that in a recent report a direct interaction between the L and N proteins in another negative-

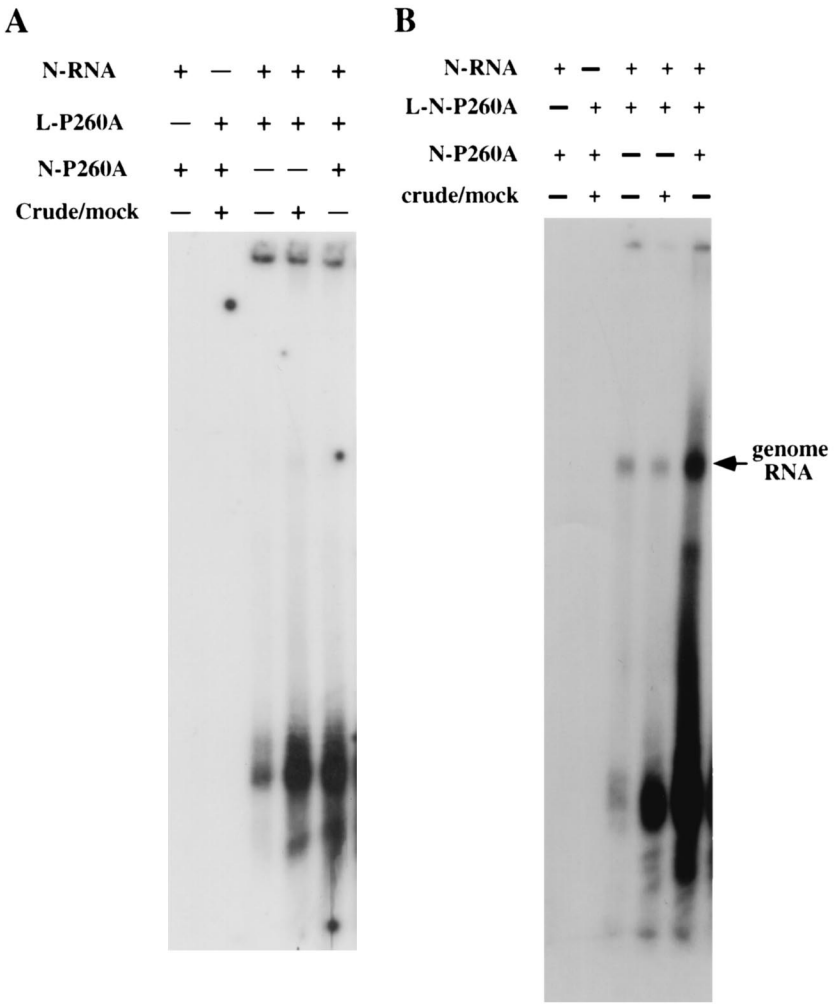

FIG. 5. Reconstitution of in vitro replication reaction using Sf21-expressed wild-type L and N proteins and the mutant P protein, P260A. ^{32}P-labeled in vitro-synthesized RNA products were analyzed as described in the legend for Fig. 4. The L-P260A (A) or L-N-P260A (B) extracts used here were partially purified by passing through a phospho-cellulose column equilibrated with a 150 mM NaCl salt concentration and combining the fractions eluted between 350 to 600 mM NaCl. The eluted fractions were rechromatographed through a second phospho-cellulose column following the same cycle. This time the peak fraction containing L, N, and P260A that eluted at an approximate salt concentration of 500 mM was used for the in vitro replication assay. Partial purification of N-P260A was achieved by loading the N-P260A-expressing S-100 extract on a phospho-cellulose column equilibrated at a 250 mM NaCl salt concentration. The flowthrough fraction was then loaded onto a DE-52 column at the same salt concentration. The eluted fraction from the DE-52 column between 400 and 600 mM NaCl was used as a purified fraction of N-P260A. Mock/crude, uninfected Sf21 extract.

strand RNA virus, human parainfluenza virus type 2, was reported (20). In view of our present observations, it can be envisaged that in that system, the L-N complex presumably represents the replicase complex, and in the presence of the N-P complex the replication reaction is further augmented. Additional experiments are needed to probe into this possibility.

Finally, based on our results, we propose that the transcriptase and replicase are structurally distinct entities with different compositions and functions. The transcriptase holoenzyme is composed of L protein bound to phosphorylated P protein oligomer (9, 13, 14) and specific host factors (10, 16) and is primarily involved in the synthesis of capped mRNAs, beginning sequentially with N mRNA synthesis and so on. The replicase, on the other hand, is composed of L protein bound to the N-P complex, forming a tripartite complex of unknown composition, and it initiates the synthesis of uncapped leader RNA which is concomitantly enwrapped by the N protein released from the soluble pool of N-P complex and continues to synthesize the full-length antigenomic RNA and, subsequently, the genomic RNA. This contention may explain the molecular basis of several unexplained observations and inferences made in earlier studies, such as (i) the leader RNA is uncapped with a polyphosphate 5′ end, whereas the mRNAs are capped (1, 8). Since it appears that replicase is structurally different from transcriptase, it may not manifest the capping activity, and thus initiates leader RNA with a polyphosphate 5′ end. Moreover, the observed differential requirements of ATP for leader RNA and mRNA synthesis (17, 25, 29) also point towards the involvement of two distinctly different polymerase

complexes. (ii) It has been previously suggested that the synthesis of leader RNA in vitro by the RNP is a "replication attempt" by the transcriptase (1, 2), and in the absence of the N-P complex, leader RNA synthesis is aborted at a specific gene junction. As per our proposition, if the leader RNA is indeed synthesized by the replicase, it must also be associated with the RNP. The presence of such a virion-associated replicase can be established by purification of the associated protein components (L, P, and N) from the RNP. (iii) The multiple initiation of RNA (leader RNA and mRNA) observed during transcription (30, 31) is, thus, carried out by two polymerase entities, replicase and transcriptase, respectively, and both must initially be located on the leader template at separate promoter sites. Recent observations, in fact, tend to support these contentions (7a, 18, 32; S. P. Whelan and G. W. Wertz, 20th Annu. Meet. Am. Soc. Virol., abstract P150, 2001). Clearly, purification of the putative tripartite complex from the insect cells as well as from the virion will be needed to gain insight into the exact polypeptide composition of the complex and delineate its precise function in the replicative reaction.

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